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Effect of Hinge Region Phosphorylation on the Localization of tHP1 in *Tetrahymena thermophila*

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Effect of Hinge Region Phosphorylation on the Localization
Of tHP1 in *Tetrahymena thermophila*

Thesis Presented by

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To the W. M. Keck Science Department

Of Claremont McKenna, Pitzer, and Scripps Colleges

In Partial Fulfillment of

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Abstract

Within the cell nucleus, there are regions of highly condensed, transcriptionally silent chromatin called heterochromatin. Heterochromatin plays an important role in both chromosomal stability and gene regulation within the cell. Heterochromatin assembly is mediated by Heterochromatin Protein 1 (HP1) binding to epigenetically marked histone tails, most notably methylated H3K9. HP1 is post-translationally phosphorylated at serine and threonine residues, and this phosphorylation has been shown to increase HP1's binding affinity for methylated H3K9 and heterochromatin formation. To study the effect of phosphorylation on heterochromatin assembly and HP1 localization within the nucleus, the unicellular protozoan *Tetrahymena thermophila* was used. *Tetrahymena* is an ideal model for this work because cells have a dynamic chromatin environment. *Tetrahymena* have an HP1-like protein, tHP1, which localizes to transcriptionally silent chromatin bodies within the otherwise transcriptionally active macronucleus. tHP1 is known to be phosphorylated at threonine-64 (site one) and at either serine-102 or threonine-103 (site two). Previous work shows that when phosphorylation at both sites is prevented, tHP1 exhibits decreased localization to chromatin bodies. In order to determine which site of phosphorylation accounts for tHP1's localization to regions of heterochromatin, mutant proteins that allow phosphorylation at only one of the two sites were generated. The efforts to engineer a mutant protein that cannot be phosphorylated at site two and to visualize the protein's localization throughout cell development are discussed. When phosphorylation is prevented at site two, tHP1 localization to regions of heterochromatin remains intact. These results suggest that phosphorylation at site one, not site two, may be responsible for tHP1 localization to macronuclear chromatin bodies. A mechanism by which site one phosphorylation influences tHP1 targeting to regions of heterochromatin is proposed. Furthermore, bioinformatics techniques are employed to identify other tHP1-like proteins within *Tetrahymena*. Characterization of these proteins will likely contribute to a more complete model of how heterochromatin is assembled in *Tetrahymena*.

Introduction

Chromatin

In all eukaryotic cells, genetic information is stored in the nucleus in the form of DNA. The DNA is associated with nuclear proteins that package it into a smaller volume so that it can fit neatly into the nucleus. This complex of DNA and proteins is called chromatin. To condense, DNA is wrapped around many nucleosomes (Figure 1). Nucleosomes are histone octamers that contain two of each of the core histones H2A, H2B, H3, and H4 (Stewart *et al.*, 2005). The C-terminal two-thirds of the core histone amino acid sequences fold to form a globular region in the proteins. The eight globular regions interact with each other to form a very stable octamer and bind DNA to the nucleosome surface (Elgin & Grewal, 2003). 147 base pairs of DNA are wrapped in two left-handed turns around each nucleosome, and histone H1 binds to the DNA where the DNA enters and exits from association with the nucleosome (Kouzarides, 2007, Elgin & Grewal, 2003). There are additional non-histone proteins that are associated with DNA that help to stabilize and organize the chromatin structure and regulate its functions (Kouzarides, 2007).

There are two forms of chromatin that can be distinguished - euchromatin and heterochromatin (Figure 1). In euchromatin, the chromatin is loosely packed and the DNA is gene rich and generally transcriptionally active.



Figure 1. DNA is wrapped around nucleosomes to form chromatin. Euchromatin is loosely packed and transcriptionally active, whereas heterochromatin is highly condensed and transcriptionally silent.

Euchromatin replicates throughout the S phase and, when visualized during interphase, appears to be dispersed throughout the nucleus (Elgin & Grewal, 2003). In heterochromatin, the chromatin is tightly condensed and the DNA is relatively gene poor, rich in repetitive sequences, and

transcriptionally silent. Heterochromatin replicates late in the S phase and, when visualized during interphase, appears to be highly condensed due to its intense staining (Elgin & Grewal, 2003).

Heterochromatin Localization, Characteristics, and Assembly

The main chromosomal targets of heterochromatin formation are regions that contain a high density of repetitive DNA elements. These include clusters of satellite sequences and transposable elements, which are found mainly at the centromeres and telomeres of chromosomes (Grewal & Jia, 2007). Formation of heterochromatin in these regions is important for chromosome integrity. For example, heterochromatin represses recombination and also protects against transposable elements (Grewal & Jia, 2007). Heterochromatin in these regions is also important for the regulation of chromosomal processes because it serves as a self-assembling domain that can recruit effector proteins (Shimada & Murakami, 2010; Kwon *et al.*, 2011). Centromeric heterochromatin is necessary for proper sister chromatid cohesion, chromosome alignment, and segregation during cell division (Bernard *et al.*, 2001; Kellum & Alberts, 1995; Obuse *et al.*, 2004). Heterochromatin can also be formed in dynamic chromatin regions in response to cellular signals (Grewal & Jia, 2007).

One of the primary characteristics of heterochromatin is gene silencing. Silencing occurs when heterochromatin spreads across domains and causes epigenetic repression of nearby gene sequences (Cavalli & Paro, 1998). Two key observations led to the conclusion that heterochromatin is able to propagate and influence gene expression in a region-specific, sequence-independent manner (Grewal *et al.*, 2007). First, in mammals, X chromosome inactivation occurs via condensation of one randomly selected X chromosome to regulate gene dosage in females. This heterochromatic structure, the Barr Body, is clonally inherited in the future cell generations. On the

active X chromosome, many genes are expressed; however, on the inactivated X chromosome, heterochromatin propagates and induces silencing (Grewal & Elgin, 2002). A similar example of clonally inherited silencing has been identified in a phenomenon called position effect variegation (PEV) in *Drosophila*. In PEV, silencing by heterochromatin is not gene specific, but rather is based on the location of that gene within the chromosome. PEV occurs following chromosome rearrangements in which one breakpoint is located within heterochromatin and a gene normally located in euchromatin is relocated to a region of heterochromatin. For example, after a chromosome rearrangement in flies that places the white locus (necessary for red eye pigmentation) within 25 kb of a heterochromatic breakpoint, variegated eye color patterns are observed in the developed flies and their offspring (Muller, 1930). This phenotype is a result of the white locus genes being silenced in many cells. PEV suggests that heterochromatin can spread along the chromosome when a normal heterochromatin barrier is removed by chromosome rearrangement (Bannister *et al.*, 2001). In each of these examples, heterochromatin is clonally inherited throughout generations, regardless of the underlying DNA sequence.

There are a distinct set of biochemical markers that characterize heterochromatin and result in heterochromatin assembly, silencing, and spreading. Heterochromatin is biochemically distinct from euchromatin due to the different epigenetic markers found on its histone tails and DNA (Stewart *et al.*, 2005). The N-terminal tails of histones emerge from the histone core and are subject to a number of post-translational modifications that modify specific amino acids residues by the addition of small chemical functional groups (Sims *et al.*, 2003). Examples of possible histone post-translational modifications include acetylation, methylation, phosphorylation, and ubiquitination (Lomberk *et al.*, 2006; Stewart *et al.*, 2005). Euchromatin is characterized by histone hyperacetylation and methylation of histone H3 at lysine 4 (meH3K4). Conversely, heterochromatin

is characterized by histone hypoacetylation in all eukaryotes and methylation of histone H3 on lysine 9 (H3K9) and/or histone H3 on lysine 27 (H3K27) in higher eukaryotes (Elgin & Grewal, 2003). Furthermore, the DNA is subject to epigenetic methylation by DNA methyltransferases (DMTs) (Elgin & Grewal, 2003). The cytosines in euchromatic DNA are hypomethylated, characteristic of transcriptionally active DNA. However, the cytosines in heterochromatic DNA are hypermethylated, characteristic of transcriptionally silent DNA. These distinguishing post-translational modifications to the histone tails and DNA are thought to be part of an intricate epigenetic mechanism that organizes genomes into distinct chromatin domains (Grewal & Jia, 2007).

One current model of heterochromatin assembly is based on the pattern of epigenetic markers on the N-termini of the histone tails that emerge from the nucleosomes (Figure 2). According to this model, specific proteins will associate with certain histone tail modifications. The recruitment of these different proteins to specifically marked regions of chromatin may activate or repress transcription or serve to maintain a specific chromatin structure (Stewart *et al.*, 2005). Histone deacetylase proteins (HDACs) deacetylate the core histone tails at specific sites. The HDACs work in conjunction with a histone methyltransferase (HMT) that specifically methylates H3K9 or H3K27, which are both associated with transcriptional repression. Methylated H3K9 or H3K27 becomes a target for binding by Heterochromatin Protein 1 (HP1). In *Drosophila*, the H3K9

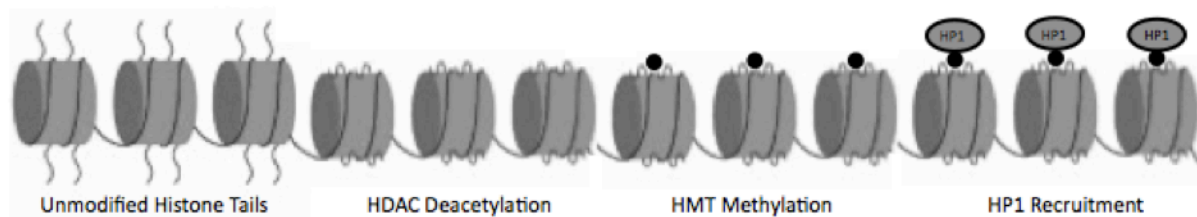


Figure 2. In the model of heterochromatin assembly based on the pattern of epigenetic markers on histone tails, unmodified histone tails are deacetylated by HDACs and methylated by HMTs. Methylation is shown by small black circles. HP1 is recruited to the methylated histone tails. Adapted from Elgin & Grewal (2003).

methyltransferase SUV39H1 recruits HP1 via histone methylation at H3K9 and also a direct protein-protein interaction between HP1 and SUV39H1 (Elgin & Grewal, 2003). Neither the methylation at H3K9 nor the SUV39H1 protein alone are sufficient to recruit HP1 (Stewart *et al.*, 2005).

Methylation at H3K9 or H3K27 and HP1 are both necessary for the formation of heterochromatin (Grewal, 2007). The epigenetic markers of heterochromatin are inherited and cause the packaging state to be maintained throughout cell replication and mitosis (Grewal & Elgin, 2003; Grewal & Jia, 2007).

Heterochromatin Protein 1 Functions

HP1 is the most widely studied heterochromatin-associated protein. HP1 was originally identified in *Drosophila*, and belongs to a highly conserved protein family with homologs found in numerous species, from yeast to humans (Grewal & Jia, 2007). Most eukaryotes and all mammals have at least three isoforms of HP1-like proteins, all of which repress euchromatic gene expression in transcriptional assays (Kwon *et al.*, 2011; Smothers & Henikoff, 2000).

HP1 plays an important role in the stability of chromosomal regions of heterochromatin. For example, HP1 is required for centromere stability in many different organisms. Centromeres are specialized regions of eukaryotic chromosomes that direct sister chromatid segregation during mitosis and serve as the platform upon which kinetochore proteins are assembled (Dialynas *et al.*, 2008). HP1 localizes to the periphery of the centromere and assists with centromere function. In *Drosophila*, a reduction in levels of HP1 causes chromosome segregation defects in which the cells possess disorganized spindles and misaligned chromosomes (Kellum & Alberts, 1995). In mammals, reduction in HP1 produces chromosome segregation defects and increased genomic instability (Obuse *et al.*, 2004). Additionally, HP1 is necessary for telomere stability in many different

organisms. Telomeres are defined by G-rich repetitive sequences, such as retrotransposons, that are elongated by telomerase and protected from degradation via a capping complex. HP1 is involved in the formation of a specialized chromatin structure at the telomeres that allows the cell to distinguish the natural chromosome end from a double-stranded DNA break. HP1 localizes to the telomeres through direct interactions with DNA and plays a role in end capping and length stability (Perrini *et al.*, 2004).

HP1 plays a complex role in gene expression; it can be either a negative or positive regulator. To repress gene expression, HP1 associates with the promoter regions of genes and prevents transcription initiation by establishing a heterochromatin environment. HP1 is necessary for the formation of heterochromatin and, thus, plays an important role in the silencing associated with X chromosome inactivation and PEV. For example, increased levels of HP1 in mice exhibit increased silencing of variegating genes near centromeric heterochromatin (Dialynas *et al.*, 2008). In addition, mammalian HP1s, HP1 α , HP1 β , and HP1 γ have each been shown to silence transcription when directly tethered to DNA via heterochromatin formation (Nielsen, 2001).

HP1 can also act as a positive regulator of gene expression for genes located within heterochromatin or that directly associate with HP1 in euchromatin. Though heterochromatin is very gene poor in comparison to euchromatin, bioinformatics has led to the discovery of several hundred genes from a variety of organisms that naturally reside within heterochromatin (Yasuhara & Wakimoto, 2006). In *Drosophila*, HP1 is required for the proper expression of several heterochromatic genes (Yasuhara & Wakimoto, 2006; Dialynas *et al.*, 2008). There are also over 200 sites in *Drosophila* euchromatin that contain methylated H3K9 and associate with HP1. Low levels of HP1 correlate with decreased levels of gene expression, indicating that HP1 is required for gene expression for euchromatin HP1-associated genes (Cryderman *et al.*, 2005).

Dysfunction in the nuclear processes in which HP1 participates, including chromosomal stability and gene expression, are related to many human diseases, including cancer. The development and progression of cancer is accompanied by changes in gene expression, including that of HP1. A review article published by Dialynas *et al.* (2008) clearly presents evidence linking HP1 to the progression of various forms of cancer. Decreased levels of HP1 expression in humans are associated with the progression of breast, brain, colon, and ovarian cancers, as well as leukemia and papillary thyroid carcinoma. For patients with embryonal brain cancer or papillary thyroid carcinoma, a reduced level of HP1 α mRNA is among the best predictors of treatment failure. In breast cancer studies, low levels of HP1 have been shown to have a causal role in regulating cancer cell invasion. In metastatic breast cancer tissues, HP1 levels were decreased as much as 95% when compared with HP1 levels in primary breast cancer tumor tissues. The correlation between low levels of HP1 and increased invasiveness in metastatic cells suggests that HP1 acts as a metastasis suppressor for breast cancer. Gaining a better understanding of HP1 function and localization to regions of heterochromatin is important because it is a possible target for cancer therapy.

Heterochromatin Protein 1 Structure

All HP1 homologs share a common structure (Figure 3). A conserved chromo domain is located at the N-terminus and a conserved chromo shadow

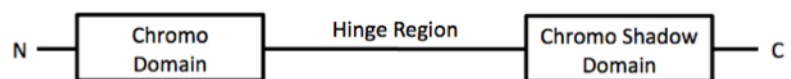


Figure 3. HP1 proteins have an N-terminal chromo domain that is connected a C-terminal chromo shadow domain by an unstructured hinge region.

domain is located at the C-terminus. These two domains are connected by a less conserved, unstructured hinge region. The chromo and chromo shadow domains form globular domains that

are closely related in primary amino acid sequence and topology (Cowieson *et al.*, 2000). Structural analysis of the HP1 homolog Swi6 in

(*Schizosaccharomyces pombe*

reveals that each domain

contains an anti-parallel, three-

stranded β -sheet and one (chromo domain) or two (chromo shadow domain) alpha helices that back against the β -sheet (Figure 4; Cowieson *et al.*, 2011). Despite their sequence similarity, the chromo and chromo shadow domains impart distinct functions to the HP1 protein.

The chromo domain binds directly to chromatin by recognizing histone tail methylation (Kwon *et al.*, 2011). Bannister *et al.* (2001) tested the specificity of the chromo domain for different sites of histone methylation and found that the chromo domain had a strong preference for methylated H3K9 (a mark of heterochromatin) but not for methylated H3K4 (a mark of euchromatin). HP1 bound equally well to di- and tri-methylated H3K9, as did all mammalian forms of HP1 (Bannister *et al.*, 2001). Furthermore, mutations of conserved residues in the HP1 chromo domain abolished HP1 binding to methylated H3K9 (Bannister *et al.*, 2001). To investigate the extent to which the chromo domain is conserved, Wang *et al.* (2000) replaced the chromo domain encoding sequence of Swi6 with that of human HP1. They found that the human chromo domain could functionally replace the yeast chromo domain, showing that chromo domain function is conserved from yeast to humans. Recent studies have provided evidence that other chromo domain containing proteins act as ATP dependent chromatin modifiers and histone H3-specific

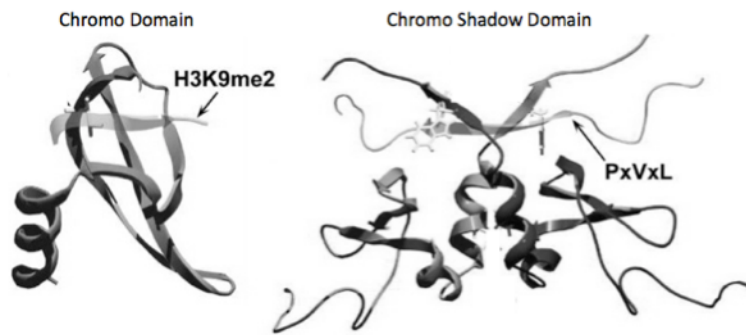


Figure 4. Ribbon diagrams constructed from the crystal structures of the chromo domain (in complex with meH3K9) and a dimer of the chromo shadow domain (in complex with PxVxL sequence). Adapted from Dialynas *et al.* (2008).

methyltransferases (Smothers & Henikoff, 2001). The chromo domain is also interacts with proteins involved in nuclear architecture and DNA replication such as the lamin B receptor protein embedded in the nuclear membrane and origin recognition complex proteins (Huang *et al.*, 1998, Ye *et al.*, 1997).

The chromo shadow domain's name derives from the fact that it is a chromo domain variant that is only found in proteins that have N-terminal chromo domains (Assland *et al.*, 1995). The chromo shadow domain mediates HP1's interactions with other proteins and with itself due to its unique ability to form dimers (Cowieson *et al.*, 2000). The formation of stable HP1 dimers via chromo shadow domain homodimerization generates a hydrophobic interaction pocket that attracts hydrophobic regions of other proteins, particularly those with a hydrophobic pentapeptide sequence. Cowieson *et al.* (2000) found that peptide pentamer binding occurs exclusively through the chromo shadow domain, and that the chromo shadow domain binds especially tightly with proteins that contain the hydrophobic sequence PxVxL (in which P is proline, V is valine, L is leucine, and x is any other amino acid). Interactions with these pentapeptide proteins provide functions such as targeting chromo shadow-containing proteins to particular sites of chromatin (Cowieson *et al.*, 2000). Furthermore, heterodimerization of the chromo shadow domain with other proteins increases the diversity of proteins that can interact with the hydrophobic pocket and, thus, with chromo shadow domain containing proteins (Cowieson *et al.*, 2000). Grewal and Jia suggest that through the chromo shadow domain's multimerization with other heterochromatic proteins, HP1 acts as the assembly platform for heterochromatin spreading and maintenance (2007).

The hinge region is the least evolutionarily conserved, varying in both sequence and length among species, and is also the least functionally understood region of HP1 (Hines, 2006). A twenty-five amino acid block in the hinge region encodes the nuclear localization signal required for active

transport of HP1 to the nucleus from the cytoplasm (Hines, 2006). It is unstructured in solution, enabling the globular chromo and chromo shadow domains to move independently of one another (Hines, 2006). It is characterized by a high density of serine, threonine, and lysine residues and contains kinase recognition sites for several different kinases (Eisenberg *et al.*, 1994). Sequence differences in the hinge region are thought to be responsible for the slightly different localizations of mammalian HP1 isoforms. The hinge region interacts with RNA, DNA, and chromatin without sequence specificity, and also interacts with histone H1 (Zhao *et al.*, 2000). Studies on the hinge region have shown that the hinge region increases the in vitro binding capacity of the HP1 chromo domain for several proteins, suggesting that the hinge region either cooperates with the chromo domain or contributes to its binding stability. Furthermore, the only artificially truncated forms of HP1 that localize to heterochromatin contain at least one chromo domain and also include a substantial portion of the hinge, suggesting that the hinge region contributes to HP1 targeting to heterochromatin (Smothers & Henikoff, 2001).

HP1 Phosphorylation

While studying HP1 in *Drosophila*, researchers noted that HP1 is initially synthesized in its most basic form but subsequently experiences an increasing net negative charge (Eisenberg *et al.*, 1994). To test whether phosphorylation was responsible for the increasing charge, HP1 isoforms were treated with phosphatases. Upon treatment, the proteins lost their net negative charges, suggesting that phosphorylation was exclusively responsible (LeRoy *et al.*, 2009). It has since been determined that all HP1 isoforms are subject to post translational phosphorylation at specific serine and threonine residues (LeRoy *et al.*, 2009). Phosphorylation has been observed at the N and C termini of HP1, but primarily occurs in the hinge region (LeRoy *et al.*, 2009; Kwon *et al.*, 2011).

Casein kinase II (CK2) is the most studied protein that is responsible for HP1 phosphorylation, though other kinases are also known to phosphorylate HP1 (Eissenberg *et al.*, 1994; LeRoy *et al.*, 2009). HP1 phosphorylation is associated with heterochromatin assembly and maintenance, increased HP1 binding to methylated H3K9 and localization to heterochromatin, effector protein recruitment, and chromosome stability.

Phosphorylation is not limited to newly synthesized HP1 in *Drosophila*; instead, preexisting HP1 can be phosphorylated *in vivo*. For example, in unfertilized eggs in *Drosophila*, HP1 is present in a hypophosphorylated form. Staged embryos showed identical levels of HP1 phosphorylation for up to two hours into development, but, after two hours, the embryos each had up to eight HP1 isoforms, all of which were hyperphosphorylated. The period of transition in HP1 phosphorylation exactly coincides with the developmental stage at which HP1 is mobilized to the embryonic nucleus to form heterochromatin and the time at which heterochromatin first becomes cytologically visible (Eissenberg *et al.*, 1994). This suggests that phosphorylation of HP1 is involved in embryonic development of heterochromatin. Similarly, in *Tetrahymena*, the tHP1 protein becomes hyperphosphorylated in response to starvation, which correlates with increased chromatin condensation in the macronucleus (Huang *et al.*, 1999).

Further support for the theory that HP1 phosphorylation is involved in the assembly and maintenance of heterochromatin was gained by a study that showed decreased phosphorylation of HP1 correlates with tissues in which heterochromatin is underrepresented (Eissenberg *et al.*, 1994). Eissenberg *et al.* (1994) measured levels of HP1 phosphorylation in two different tissues that contained different levels of heterochromatin. Larval imaginal discs and brain tissues are mitotically active, have high amounts of heterochromatin, and express highly phosphorylated HP1 isoforms. Larval salivary gland and fat body tissues undergo a preferential amplification of euchromatin

relative to heterochromatin and express hypophosphorylated HP1 isoforms. Furthermore, when Zhao *et al.* (2000) mutated *Drosophila* HP1 to prevent hinge region phosphorylation, heterochromatin silencing activity was highly reduced. Using a transgenic fly model, it was found that mutant HP1 caused no elevated levels of position effect silencing. This suggests that the extent to which HP1 is phosphorylated affects the incorporation of HP1 into heterochromatin (Eissenberg *et al.*, 1994).

In an experiment conducted with mouse and human cells expressing HP1 α , Hiragami-Hamada *et al.* (2011) showed that N-terminal phosphorylation leads to enhanced HP1 α binding to methylated H3K9 and is necessary for proper HP1 α localization to heterochromatin. N-terminal phosphorylation of HP1 alpha enhanced binding to not only methylated H3K9, but also to unmethylated H3 tails. This suggests that N-terminal phosphorylation secures chromo domain binding to methylated H3K9. Disruption of HP1 α phosphorylation reduced the protein's affinity for methylated H3K9 and impaired targeting to regions of heterochromatin, indicating that HP1 α phosphorylation is essential for proper localization to regions of heterochromatin. As a result of mutant HP1 α 's reduced targeting to regions of heterochromatin, increased chromosomal instability was observed. A high percentage (18 - 64 percent) of mutant cells displayed several aberrant chromosomes (Hiragami-Hamada *et al.*, 2011). This finding indicates that HP1 α phosphorylation is necessary for chromosomal stability. An additional study that supports this finding is that of Shimada and Murakami (2009). They determined that Swi6 phosphorylation is associated with recruitment of effector proteins that are necessary for sister-chromatid cohesion. This protein recruitment mechanism by HP1 is independent from that for effector proteins involved in transcription silencing. Phosphorylation of Swi6 alters the HP1 binding affinity with different proteins, which could confer different functions and localizations to the protein.

Hinge Region Phosphorylation

Phosphorylation and dephosphorylation in the hinge region is dynamic through the cell cycle and may play an important role in HP1 binding and gene silencing activity (Hines, 2006). Hinge region phosphorylation has been shown to confer different binding properties to HP1 (Shareef *et al.*, 2003; Zhao *et al.*, 2001; Badugu *et al.*, 2005). Amino acid substitutions that mimic phosphorylation (serine to glutamate) make HP1 incapable of binding to ORC1 (Origin of Recognition Complex 1) or HOAP (HP1/ORC Associated Protein), but increase HP1's ability to homodimerize and bind methylated H3K9 (Badugu *et al.*, 2005; Hines, 2006). Amino acid substitutions that mimic non-phosphorylation (serine to glycine) do not affect homodimerization or HP1's binding to HOAP, ORC1, or methylated H3K9. However, these mutant proteins completely coat the chromosome, suggesting that hinge region phosphorylation may be responsible for HP1's binding specificity to regions of heterochromatin (Badugu *et al.*, 2005; Hines, 2006).

Tetrahymena thermophila as a model system

In this study, *Tetrahymena thermophila* is used as a model organism to study the effect of hinge region phosphorylation of HP1 on its localization to heterochromatin in the nucleus. *Tetrahymena* are unicellular ciliated protozoans found in freshwater ponds (Figure 5). They are easily grown

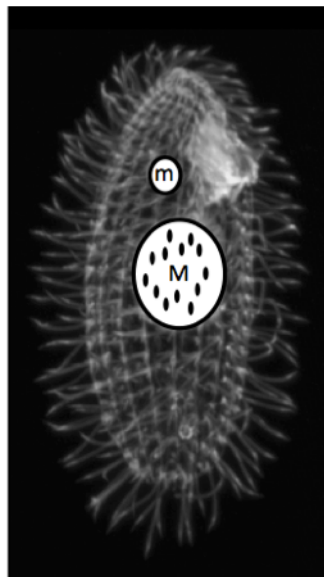


Figure 5. *Tetrahymena thermophila* have two nuclei. The micronucleus (m) is transcriptionally inactive and the macronucleus (M) is transcriptionally active. Regions of heterochromatin in the macronucleus are called chromatin bodies (shown in black).

and genetically manipulated in the laboratory, making them a good general model organism (Martindale *et al.*, 1982).

Tetrahymena is an ideal model organism for this work because, during conjugation, cells have a dynamic chromatin environment in which heterochromatin formation and HP1 localization may be studied. Throughout conjugation (*Tetrahymena's* sexual cycle), HP1 localizes exclusively to regions of facultative heterochromatin, not to regions of constitutive heterochromatin. Thus, *Tetrahymena* provide an ideal organism in which to study what role post-translational modifications, such as phosphorylation, play in the specific targeting of HP1 to different forms of heterochromatin. Furthermore, the typical methylated H3K9 epigenetic mark of heterochromatin is absent in *Tetrahymena*. This is the only known organism without this mark, so work with *Tetrahymena* is unique because HP1 recruitment and localization to regions of heterochromatin occurs via a methylated H3K9-independent pathway. The mechanism by which HP1 localizes to heterochromatin in *Tetrahymena* and the effect of post-translational modifications within this pathway is currently unknown.

Nuclear dimorphism, in which a cell has two nuclei that contain different chromatin environments, is a distinguishing feature of ciliate protozoans, including *Tetrahymena* (Figure 5). *Tetrahymena* have one large macronucleus that contains their somatic DNA. They also have a small micronucleus that contains their germ line DNA which is composed entirely of heterochromatin. The macronucleus is highly polyploid (about 45N) whereas the micronucleus is diploid (2N). The macronucleus accounts for almost all transcription within *Tetrahymena*, whereas the micronucleus is transcriptionally silent. The transcriptionally active macronucleus contains euchromatin; however, there are distinct regions of condensed chromatin within the macronucleus called chromatin bodies (Figure 5). Using ultrastructural analysis, it was shown that a large percentage of

the *Tetrahymena* genome exists in these transcriptionally silent chromatin bodies (Dorman, unpublished).

These distinct areas of heterochromatin and euchromatin undergo dynamic structural changes during starvation and conjugation that may be observed via microscopy (Cole & Sugani, 2012). During starvation, *Tetrahymena* stop dividing vegetatively. This change is accompanied by a reduction in macronuclear size, an increase in the size of the chromatin bodies, a decrease in overall gene transcription, and the expression of a unique set of starvation-induced genes (Huang *et al.*, 1998; Jeter *et al.*, 1974).

During conjugation, two cells pair and the micronucleus from each cell undergoes a round of meiosis and mitosis, leading to two identical haploid micronuclei in each cell (Figure 6). The conjugation partners reciprocally exchange micronuclei, and subsequent micronuclear fusion forms one diploid micronucleus in each cell. These diploid micronuclei undergo mitosis to form two new macronuclei and two micronuclei in

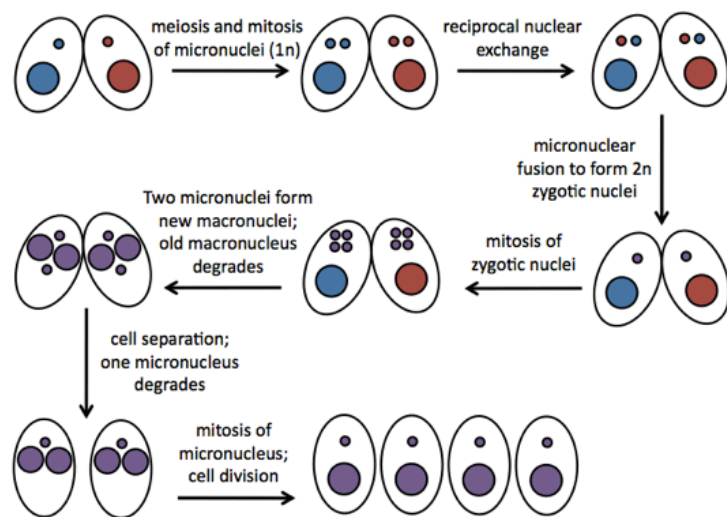


Figure 6. During conjugation, *Tetrahymena* cells exchange haploid micronuclei (blue and red) that merge to form a diploid micronucleus (purple). The micronuclei undergo mitosis to form two new micronuclei and two new macronuclei, leading to a dynamic chromatin environment within the cell. Adapted from Matzke *et al.* (2005).

each cell, at which point the old macronucleus degrades. The cells then separate, one micronucleus degrades in each cell, and the cells divide to yield four daughter cells (Matzke *et al.*, 2005).

Throughout the conjugation process, chromatin condensation in the macronucleus may be

observed (Figure 6). Observation of HP1 localization throughout the formation of heterochromatin provides a unique opportunity in which to better characterize the role of HP1 in chromatin dynamics.

tHP1 Protein in Tetrahymena thermophila

Tetrahymena and humans share many homologous genes, one of which is the HP1-like protein tHP1 (Huang *et al.*, 1998). In 1998, Huang *et al.* published their discovery of the 28-kDa tHP1 protein. Structural similarities between tHP1 and *Drosophila* HP1 were quickly noted, including the presence of chromo and chromo shadow domains within the protein and its association with regions of heterochromatin within an otherwise transcriptionally active environment. They found that tHP1 is missing from the transcriptionally silent micronuclei of *Tetrahymena*, instead localizing to chromatin bodies within the otherwise transcriptionally active macronucleus. This finding supported the theory that some HP1-like proteins are involved in localized transcriptional repression. Unlike in *Drosophila*, loss of the tHP1 protein is not lethal in *Tetrahymena*; however, during starvation, cells that lack tHP1 display decreased rates of survival (Huang *et al.*, 1998).

Like other HP1 proteins, tHP1 is subject to post-translational phosphorylation that influences the protein's function and localization (Huang *et al.*, 1998). Previous research from the Wiley Lab shows that tHP1 is subject to phosphorylation during both starvation and conjugation (Figure 7). An acid urea gel run with tHP1 from various stages of the cell cycle, including vegetative growth, starvation, and various intervals of time into conjugation shows that tHP1 is phosphorylated up to two times during starvation and up to five times during conjugation (Wiley, unpublished). These changes in phosphorylation correlate with dynamic changes in the chromatin

environment within the cell. The increase in phosphorylation correlates with the differentiation of micronuclei into new macronuclei, called anlagen. At this point, regions of heterochromatin are formed within the newly differentiated macronucleus.

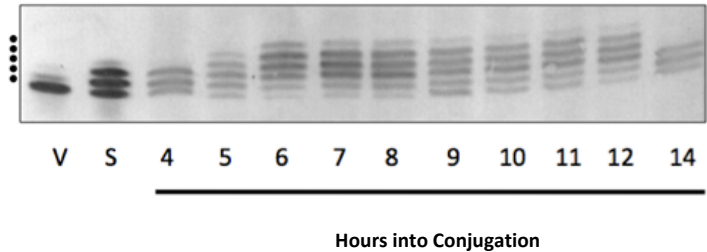


Figure 7. An immunoblot with α-tHP1 on an acid urea gel. Gel was run with tHP1 from vegetative (V), starved (S), and conjugating cells indicates that tHP1 is phosphorylated during starvation and conjugation. Each band denoted with a circle indicates the presence of a phosphoryl group.

In order to determine the sites of this phosphorylation on the protein, phosphorylated tHP1 was subjected to mass spectrometry. Two sites of phosphorylation were identified, and both were located in the hinge region of the protein (Figure 8; Wiley, unpublished). One site of phosphorylation is the threonine at amino acid position 64. Another site of phosphorylation is either the serine at amino acid position 102 or the threonine at amino acid position 103. Previous work in the Wiley lab has shown that, when both sites of phosphorylation are mutated, tHP1 shows decreased localization to chromatin bodies during starvation (Wiley, unpublished). This finding suggests that hinge region phosphorylation imparts necessary localization properties to the protein.

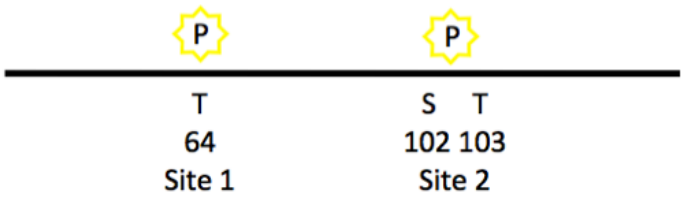


Figure 8. Mass spectrometry revealed that tHP1 is phosphorylated at two sites in the hinge region.

In this work, we engineered a mutant tHP1 protein that could not be phosphohrylated at site two. This mutant protein was expressed and visualized in *Tetrahymena*. Because wild type and mutant tHP1 display comparable macronuclear localization while double mutant tHP1 displays aberrant localization, we propose a model by which phosphorylation at site one alone affects

heterochromatin formation. Furthermore, to develop a more complete model of heterochromatin formation in *Tetrahymena*, bioinformatics techniques were used to identify other chromo domain containing proteins and characterize their relationships to tHP1. Statistical analysis revealed two proteins that are closely related to tHP1, and protein expression profiles indicate that they both are active during dynamic chromatin changes during conjugation. We suggest that these two proteins should be studied further to determine their role in chromatin formation.

Studying tHP1 in *Tetrahymena* not only leads to better understanding of the functions of this particular protein, but, because tHP1 is closely related to HP1 proteins in other organisms, it also leads to a broader understanding of the role of HP1 in the formation of heterochromatin and gene regulation in other organisms.

Methods

Site Directed Mutagenesis

The Stratagene QuikChange II XL Site-Directed Mutagenesis Kit was used to introduce mutations into the tHP1 gene (Figure 9). A pENTR vector containing kanamycin resistance and the wild type tHP1 gene was obtained from previous student research in the Wiley lab (Figure

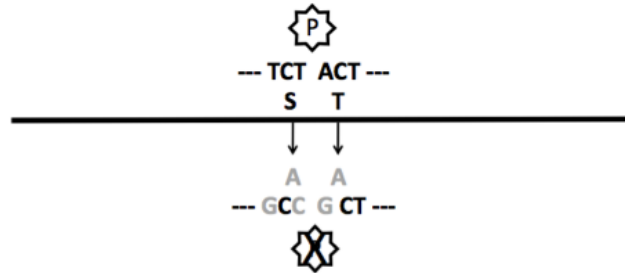


Figure 9. Using site-directed mutagenesis, the sequence coding for serine at amino acid position 102 and threonine at amino acid position 103 will be mutated to code for two alanines.

10). Two complementary oligonucleotide primers containing the desired mutation, flanked by unmodified nucleotide sequences, were obtained from previous student work and used in a thermal cycling reaction (Table 1).

A sample reaction and a control reaction were prepared for thermal cycling. The sample reaction was prepared in a PCR tube, using 5 μ L 10X reaction buffer, 10 ng of the pENTR plasmid, 125 ng forward primer containing the mutations, 125 ng reverse primer containing the mutations, 1 μ L of dNTP mix, 3 μ L QuickSolution reagent, and double distilled water to a final volume of 50 μ L. The control reaction was prepared in the same way, but with 10 ng pWhitescript 4.5 kb control plasmid (5 ng/ μ L), 125 ng of the forward oligonucleotide control primer, and 125 ng of the reverse

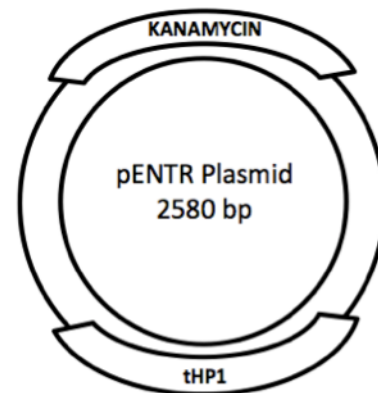


Figure 10. The pENTR plasmid contains wild type tHP1 and kanamycin resistance.

oligonucleotide control primer. To each reaction, 1 µL of pfuUltra HF DNA polymerase (2.5U/µl) was added and the reactions were immediately placed in the thermal cycler. Each reaction was brought to 95°C for one minute. The sample reaction underwent 18 cycles of 50 seconds of denaturing at 95°C, 50 seconds annealing at 60°C, and 3.1 minutes of extension at 68°C. The control reaction underwent 12 cycles of 50 seconds denaturing at 95°C, 50 seconds annealing at 60°C, and five minutes of extension at 68°C. After the cycles finished, they were held for 7 minutes at 68°C and then stored at 37°C.

Table 1. Forward and reverse primers were used to synthesize plasmids with the desired mutations. Primers contained the desired mutations (underlined) which were flanked by nucleotide sequences that were complimentary to wild type tHP1.

Forward	5' - TCTAATCCTAAAG <u>CCGCT</u> CCTAAATTAGATAAAAAAGCATTATATCCAGTGAAG - 3'
Reverse	5' - CTTACACTGGATCTAATGCITTTTTTATCTAATTTAGGAG <u>CGGCT</u> TTAGGATTAGA - 3'

To digest the nonmutated parental plasmids and isolate the newly synthesized mutated plasmids, a restriction enzyme digest was used. To each amplification reaction, 1 µl of the Dpn I restriction enzyme (10 U/µl) was added. The reaction mixtures were centrifuged for one minute and incubated at 37°C for 1 hour to digest the nonmutated parental dsDNA.

To amplify the mutant pENTR plasmid, the mutated plasmids were chemically transformed into *E. coli* cells. For each control and sample reaction, 45 µl aliquots of XL10-Gold ultracompetent cells were used. Two µl of the provided β-ME mix and 2 µl of the DpnI treated DNA from each control and sample reaction were added to the separate aliquots of the ultracompetent cells. To verify the transformation efficiency of the XL10-Gold ultracompetent cells, 1 µl of 0.01 ng/µl pUC18 control plasmid was added to another 45 µl aliquot of ultracompetent cells. The reactions were incubated on ice for 30 minutes. The cells were heat-pulsed in 42°C water for exactly 30 seconds and were immediately incubated on ice for 2 minutes following heating. To each tube, 0.5 ml of 40°C NZY+ broth (see preparation below) was added and the tubes were incubated for 1 hour at

37°C and shaking at 225 rpm. 250 µl of the sample reaction were plated on an LB + kanamycin (50 µg/ml) agar plate. 250 µl of the pWhitescript mutagenesis control were plated on an LB + ampicillin (100 µg/ml) agar plate. Five µl of the pUC18 transformation control were added to 200 µl NZY+ broth and were plated on an LB + ampicillin (100 µg/ml) agar plate. This plate was prepared for blue-white color screening by spreading 100 µl of 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 100 µl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) 30 minutes prior to plating. The plates were incubated at 37°C for 24 hours.

NZY+ broth was prepared by dissolving casein hydrolysate (10 mg/ml), yeast extract (5 mg/ml), and NaCl (5 mg/ml) in deionized waer. The pH was adjusted to 7.5 using 10N NaOH. Following autoclaving, filter sterilized MgCl₂ (final concentration 1.2 g/ml) and MgSO₄ (1.5 g/ml) and glucose (final concentration 3.5 g/ml) were added.

Mutant tHP1 pENTR Plasmid Purification

Liquid cultures were made for six *E. coli* colonies from the LB + kanamycin plate to amplify the mutant tHP1 pENTR plasmid. They were grown in 5 ml of LB + kanamycin (100 µg/ml) broth. The cultures were incubated at 37°C and 150 rpm for 24 hours.

To purify the mutant plasmids from the six *E. coli* cultures, the Quagen QIAprep kit and protocol were used. The six cultures were centrifuged and 3.0 ml worth of pelleted *E. coli cells* from each were resuspended individually in 250 µl Buffer P1. Then, 250 µl Buffer P2 were added to each tube to open the cells and denature the plasmid DNA, genomic DNA, and proteins. To adjust the pH to 7 and allow the RNA and plasmids to reseal and stay soluble in solution, 350 µl of Buffer N3 were added to each tube. This mixture was centrifuged for 10 minutes at 16,000 rcf until compact white pellets formed at the bottom of each tube. The supernatant from each was applied

to a QIAprep spin column and the columns were centrifuged for 1 minute at 16,000 rcf. The columns were first washed with 0.5 ml buffer PB and then washed with 0.75 ml Buffer PE. Residual wash was removed by centrifuging for 5 minutes at 16,000 rcf. The six QIAprep columns were placed in six clean 1.5 ml microcentrifuge tubes. The DNA was eluted from the columns by adding 50 µl Buffer EB, letting the columns stand for 1 minute, and centrifuging for 1 minute at 16,000 rcf. Spectrometry was used to determine the DNA concentrations for each of the six samples.

Sequencing Reaction

The six samples of purified DNA were sent to the Rancho Santa Ana Botanic Gardens for sequencing with an Applied Biosystems 3100 Genetic Analyzer. The mutant tHP1 sequence on the pENTR plasmid was cycle-sequenced in a PCR reaction with 3' dye labeled dideoxynucleotidetriphosphate (ddNTP) terminators (each ddNTP was labeled with a unique fluorescent compound), AmpliTaq DNA polymerase, and a primer designed to target the mutated region of the template strand (Figure 11). The reaction mixture included the pENTR mutant plasmid to be sequenced, 1.6 pmol forward primer, 2 µl ABI terminator mix (buffer, dideoxynucleotide terminators, and AmpliTaq), 3 µl ABI buffer, and deionized water to 10 µl final volume.

The PCR reaction involved 35 cycles of denaturing for 30 seconds at 96°C, annealing for 15 seconds at 50°C, and extension for 4 minutes at 60°C.

Following the cycle-sequencing, samples were purified using Princeton

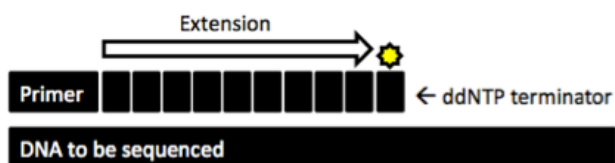


Figure 11. During PCR, primers were extended by the incorporation of deoxynucleotide-triphosphates. Fluorescently labeled dideoxynucleotidetriphosphates (ddNTPs) were incorporated at random and terminated the chains.

Separation spin columns, transferred to a sample plate, and run immediately on the 3100 Genetic Analyzer. During gel electrophoresis, a laser scanned each of 16 capillaries causing any dye particles in its path to fluoresce. The strength and color of the fluorescence was recorded and subsequently analyzed to assign a base to each peak. The resulting data were presented in the form of a chromatogram. The chromatogram showed the heights of each dye peak throughout the length of the gel and the base identity assigned to each peak.

Purification of Mutant tHP1-containing pENTR Plasmid

To amplify a successfully mutated tHP1 pENTR plasmid, a liquid culture was grown following the procedure above. To isolate and purify a large amount of the mutant tHP1-containing pENTR plasmid the boiling method and a subsequent ethanol precipitation were used.

The culture was divided between three 1.5 ml tubes and centrifuged for 5 minutes at 16,000 rcf. Pelleted *E. coli* cells were resuspended in 350 µl of Sucrose Lysis Buffer (0.75 M sucrose, 1% Triton-X 100X, 40 mM EDTA, 50 mM Tris [pH 8.0], sterile water). To each tube, 25 µl of Lysozyme (10 mg/ml in TE) were added and the tubes were incubated at room temperature for two minutes. The tubes were placed in a 99°C heat bath for exactly one minute and then centrifuged at 16,000 rcf for 15 minutes. Pellets of cell debris were removed and discarded, and 40 µl of 3M NaOAc and 220 µl of isopropanol were added to the supernatant in each tube to precipitate the DNA. The tubes were incubated at room temperature for 5 minutes and were centrifuged for 5 minutes at 16,000 rcf. The supernatant was discarded and 1 ml of 70% ethanol/30% water was added to each tube to wash the salt off the plasmid pellets. The tubes were centrifuged for 5 minutes at 16,000 rcf, and the supernatant was discarded. The pellets were allowed to completely dry at room temperature,

resuspended in 15 μ l 1X TE (pH 8.0), and combined into one tube. Spectrophotometry was used to determine the DNA concentration.

The isolated DNA was then purified using a phenol extraction and ethanol precipitation. An equal volume of phenol/chloroform/isoamyl alcohol was added to the 45 μ l of tHP1-containing pENTR plasmid. The tube was vortexed for 10 seconds and centrifuged for 2 minutes at 16,000 rcf. The top aqueous layer, containing the plasmid DNA, was pipetted into a new 1.5 ml tube and the organic layer was discarded. 135 μ l of 3M NaOAc was added to the aqueous solution. To precipitate the plasmid DNA, -20°C 100% ethanol was added and the tube was vortexed for 10 seconds and centrifuged for 2 minutes at 16,000 rcf. The sample was frozen for 24 hours at -20°C to allow all of the plasmid DNA to precipitate. After freezing, the tube was centrifuged for 5 minutes at 16,000 rcf. The supernatant was discarded and 1 ml of 70% ethanol/30% water was added to the tube to wash the salt off the plasmid pellet. The tube was centrifuged for 5 minutes at 16,000 rcf, and the supernatant was discarded. The pellets were allowed to completely dry for 15 minutes at room temperature. The DNA was resuspended in 50 μ l of sterile water. A spectrophotometer was used to determine the concentration of the purified plasmid DNA.

Recombination of tHP1 mutant into HA and GFP vectors

The human influenza hemagglutinin (HA) and green fluorescent protein (GFP) were selected as tags to be added separately to the mutant tHP1 protein. In order to add these tags to the mutant tHP1 sequence, recombination reactions were performed with the LR Clonase enzyme, which is a purified form of phage Lambda recombinase. This enzyme recognizes the coding sequence from the pENTR vector and recombines it into HA (pBM2HA-GTW) and GFP (pIGF-GTW) destination vectors (Figure 12). For the HA recombination reaction, 50 ng of the mutant tHP1-

containing pENTR plasmid and 400 ng of the HA destination vector were mixed and brought to a volume of 4 µl with sterile deionized water. For the GFP recombination reaction, 150 ng of the mutant tHP1-containing pENTR plasmid and 400 ng of the pIGF-GTW destination vector were mixed and brought to a volume of 4 µl with sterile deionized water. To each reaction, 1 µL of LR Clonase II enzyme was added. These reactions

were mixed and incubated for 24 hours at room temperature. To terminate the recombination reaction, 0.5 µl

Proteinase K solution were added to each sample and the samples were incubated at 37°C for 10 minutes.

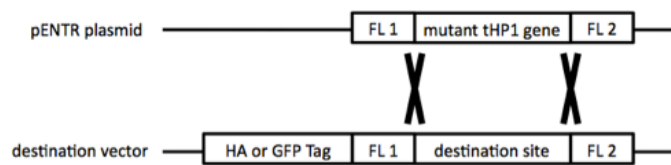


Figure 12. An ATT recombination reaction was used to incorporate the mutant tHP1 sequence into pB2MHA-GTW and pIGF-GTW destination vectors. The enzyme LR Clonase was used to cut at the indicated sites.

Chemical Transformation of Competent E. coli

To transform the recombinant HA and GFP vectors into *E. coli*, 2 µl of the HA recombination reaction DNA and 2 µl of the GFP recombination reaction DNA were added to respective aliquots of 50 µl of competent *E. coli* cells. These tubes were incubated on ice for 30 minutes and then transferred to a 42°C water bath for exactly 90 seconds. They were then immediately chilled in an ice bath for 2 minutes. 800 µl of SOC broth were added to each tube, and the cultures were incubated for 45 minutes in a 37°C water bath to allow the bacteria to recover and express the ampicillin resistance marker encoded by the HA and GFP plasmids. 200 µl of each culture were plated on LB + ampicillin (100 µg/ml) plates with sterile glass beads. The dish was incubated for 24 hours at 37°C.

Plasmid Amplification and Purification for Restriction Digest Analysis

To amplify the HA and GFP tagged mutant tHP1-containing plasmids, liquid cultures were grown for four *E. coli* colonies from the HA recombination reaction LB + ampicillin plate and four *E. coli* colonies from the GFP recombination reaction LB+ ampicillin plate. Cultures were grown using the procedure discussed above, but using ampicillin (100 µg/ml) instead of kanamycin.

To isolate the HA and GFP tagged mutant tHP1 plasmids, the boiling method (procedure followed as written above) was used for 1.5 ml of each culture. The isolated plasmid DNA was then purified using a phenol extraction and ethanol precipitation (procedure followed as written above, except DNA pellets were resuspended in 10 mM HEPES). A spectrophotometer was used to determine the concentrations of the purified plasmid DNA samples.

Restriction Enzyme Digest Analysis

Five µL of each sample of purified mutant tHP1 on the GFP vector was digested overnight with BamHI in a 20 µL digest reaction. As a positive control, pIGF-GTW vector alone was also digested with BamHI in a 20 µL digest reaction. Gel electrophoresis was used to determine if the fusion plasmid was created. Samples and 1 kb ladder were loaded in an agarose gel (with ethidium bromide). Band sizes were compared with published band sizes expected for digested GFP vector alone and for digested GFP vector with gene of interest.

Transformation of GFP-tHP1 mutant to Tetrahymena thermophila

Thirty ml cultures of CU427 and CU428 were centrifuged in a swing bucket rotor at 2862 rcf for three minutes. The supernatant was removed immediately and the cells were resuspended in 35 ml of 10mM Tris (pH 7.5). Cell densities were adjusted to $2-4 \times 10^5$ cells/ml. Resuspended

CU427 and CU428 were mixed together, poured into sterile Petri dishes, and placed in a 30°C incubator at 150 rpm. After 16 hours of starvation, rotation stopped to allow mating.

After 9 hours of mating, cells were centrifuged at 670 rcf for three minutes, the supernatant was decanted, and the cells were resuspended in 40 mL of 10 mM HEPES. After five minutes, the cells were centrifuged again at 670 rcf for three minutes and the supernatant was removed. The cells were resuspended in 400 µl of 10 mM HEPES. A mixture of 200 µl of cells and 15 µl of DNA in 10 mM HEPES (15 µg mutant tHP1 on GFP vector) was pipetted into a sterile 4 mm gap electroporation cuvette. The cuvette was electroshocked once at 225 V, 25 Ω, and 175 µF, and the time constant was recorded. Cells were washed immediately from cuvette by adding 500 µl growth medium and transferred to a flask containing 30 ml 1X SPP media with 250 µL 1X PSF (penicillin, streptomycin, and fungizone) and then placed in a 30°C incubator overnight. The same procedure was followed for cells after 10 hours of mating.

The next day, both samples of cells were plated with paromomycin (PRM) to a final concentration of 90 µg/ml to select for transformants. 150 µl was plated into the wells of two sterile 96-well plates for each sample, and the plates were placed in a 30°C incubator. After four days, 5 µl of cells from the wells with the most growth were transferred to a new liquid culture of 1X SPP, 1X PSF, and 90 µg/ml PRM.

Fluorescence Microscopy

To examine transformed cells for GFP expression and localization, cell cultures were grown to mid-logarithmic phase (1.8×10^5 cells/ml) overnight in 1X SPP media with 1X PSF. Cells were induced with CdCl₂ to a final concentration of 1 µg/ml three hours before examination.

Cultures of 5 μ l were centrifuged at 1500 for two minutes, and the supernatant was removed immediately with a P-200 pipet. This centrifugation was repeated and remaining supernatant was removed immediately with a P-10 pipet. 1 μ l of 20 μ g/ml DAPI was added to the cells. After five minutes, 2 μ l of cells was added to 5 μ l of 2% methylcellulose on a microscope slide, and a coverslip was placed on top. The cells were observed by fluorescence microscopy at 100X magnification on a Nikon Eclipse E400 fluorescence microscope with a Nikon HBO 100 watt power source for illumination.

Construction of a Phylogenetic tree for Tetrahymena Chromo Domains

Bioinformatics tools were used to build a phylogenetic tree of the chromo domains found in *Tetrahymena* and those from the well-characterized HP1 proteins in yeast (Swi6), the fruit fly (HP1a), and humans (HP1 α). From the *Tetrahymena* Genome Database Wiki (TGD), the genomic, coding, and amino acid sequences for tHP1 (Gene ID: TTHERM_00705240) were obtained. Using the protein sequence, the chromo domain sequence in tHP1 was identified and isolated. This sequence was used in a BLAST (Basic Local Alignment Search Tool) search in the TGD to identify proteins with a related chromo domain. Then, the TGD was searched for additional chromo domain-containing proteins by searching with “chromo domain” in the search bar. All of these identified protein sequences were assembled into a document in FASTA format and were run individually through the Pfam Database to determine whether they had identifiable chromo domains, and, if so, the score in bits, their e-values, and alignments.

Similarly, using the tHP1 chromo domain protein sequence, homologs were identified in yeast (from the *Schizosaccharomyces pombe* Genome Database), fruit flies (NCBI *Drosophila melanogaster* Genome Database), and humans (NCBI *Homo sapiens* Genome Database) using a BLAST search on the NCBI database. All of these identified protein sequences were assembled into

a document in FASTA format and were run individually through the Pfam Database to determine whether they had identifiable chromo domains, and, if so, the score in bits, their e-values, and alignments.

To create a FASTA format chromo domain alignment, the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program from the European Bioinformatics Institute was used. To determine the phylogenetic relationships, the FASTA formatted multiple alignment was entered into the PhyML program from the Lirmm Laboratory's *Méthodes et Algorithmes pour la Bio-informatique*. The results were obtained in the Newick format. Then, the Newick formatted tree was entered into the TreeDyn program to create a graphical representation of the tree.

Gene expression profiles were obtained for closely related proteins from the Tetrahymena Genome Database and the Tetrahymena Functional Genome Database.

Results

tHP1 Site 2 Mutant Cloned onto pENTR Plasmid

To construct a mutant tHP1 gene sequence in which Site 2 (serine 102 and threonine 103) could not be phosphorylated, site directed mutagenesis was used. The DNA sequence coding for serine and threonine residues (TCT ACT) was mutated to code for two alanine (GCC GCT) residues. Alanine was chosen because it is a small, non-polar amino acid that likely does not impart major structural or functional changes to the protein and cannot be phosphorylated.

Sanger sequencing was used to determine whether the desired mutations were successfully incorporated into the tHP1 gene on the pENTR plasmid. The chromatogram received from the Rancho Santa-Ana Botanical Gardens revealed that the mutagenesis was successful (Figure 13). Clear, unambiguous peaks were visible for each base that was mutated in the tHP1 gene. Each peak represents the strength and color of the fluorescence that was recorded, and each peak was assigned a base identity. The sequencing

Residue: 375 to 380				
121	AATAGTAATC	CCTTACAATT	ACATCAATTA	ATAAATCAAC
161	CATAATTTAT	TAATTAATAT	AAGCAAAATG	TTGAATAAAT
201	TTTTTAACCT	CAATTTTAAT	TATATTTAGA	AAGCAATTAC
241	TTAGCAATGA	TAAACCTTAG	ACACCCATTA	AGAAATCAAT
281	TAAAAAAGGA	GCTTCAACTC	ATTAAACACG	TAAATCTAGC
321	TCAACTACTA	AGAGAAGTGT	TAAAAATCA	GTTGAAAAAG
361	CATCTAATCC	TAAAGCCGCT	CCTAAATTAG	ATAAAAAAGC
401	ATTAGATCCA	GTGTAAGAAA	TTTAAATAGA	AATGAGCAAA
441	CAAACCTGTTA	TACTTAAAA	TAAATTAATT	TAAATAAATA
481	GGAAGGAAAT	TCATAATTAT	TAAAGGATG	TCAGTAAACT
521	TCTTTTAAAG	AGTGATATAA	CCTCCATATT	GCACGTTTTT
561	AAGGATGATG	GAAAACTCT	TTACACAGTC	ACAATTGGAA
501	AACATCCTAA	ATAATATTTA	GTATCAAGTA	GCTAAATTGC
541	TAAGTATGCT	TCTCCCGATC	TCATAGTTAG	ATTCTATGAA
581	AAGCTAATCC	GCTGAAAGGG	TGGCGCGGCC	GACCCAGCTT
721	CTTGATACA	AAGTTTGCCA	TATTATAAAG	AAAGCATTTG
761	TATATCAATT	TTGTTGCAAC	GAACAGGTCA	CTATCAGTCA
801	AAATAAAATC	ATTATTTGCC	ATCCAGCTGA	TATCCCTAT
841	AGTGAGTCGT	ATTACATGGT	CATAGCTGTT	TCCTGGCAGC
881	TCTGGCCCGT	GTCTCAAAAT	CTCTGATGTT	ACATTGCACA
921	AGATAAAAT	ATATCATCAT	GAACAATAAA	ACTGCTCTGCT
961	TACATAAACA	GTAATACAAG	GGGTATGAG	CCATATTCAA
1001	CGGGAAACGT	CGAGGCCGCG	ATTAAITCAC	ATGATGCTGA
1041	TTTATATGGT	ATAATGGCTC	GCGATATGTC	GGCATCAGTG
1081	CGACATCTAT	TCGCTTGTAT	GAAGTCGATG	TGTGCGTCGA
1121	AATGTTTTTC	ACTGCCAAGT	ACGTGCCCAT	GTAAGTGGT

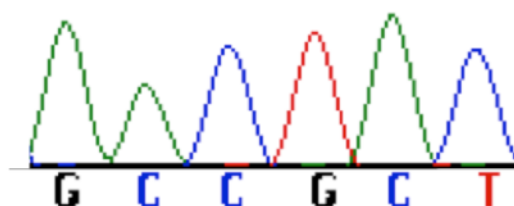


Figure 13. The Sanger sequencing chromatogram reveals that the desired GCCGCT sequence has been successfully incorporated into the tHP1 gene on the pENTR vector.

results confirm that the desired mutant sequence, GCC GCT, was obtained.

tHP1 Site 2 Mutant Cloned into GFP and HA Vectors

In order to examine the mutant tHP1 protein localization in *Tetrahymena*, the mutant was tagged with biochemical markers that can be visualized by various imaging techniques. The human influenza hemagglutinin (HA) and green fluorescent protein (GFP) were selected as tags to be added separately to the mutant tHP1 protein. The HA tag is a 27 base sequence that typically does not interfere with the bioactivity of the protein to which it is attached. Protein localization can be determined using immunofluorescence staining with a fluorescent HA antibody. The HA tag also facilitates protein isolation and purification. The GFP tag is a 238 amino acid protein that exhibits green fluorescence when exposed to ultraviolet blue light and typically does not interfere with the bioactivity of the protein to which it is attached. Live protein localization can be determined using fluorescence microscopy.

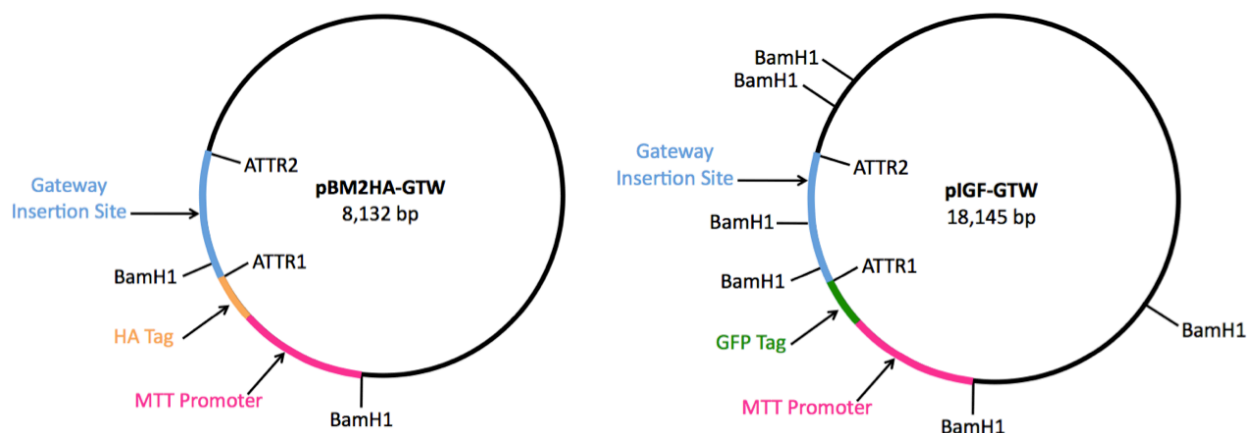


Figure 14. Mutant tHP1 was incorporated into the pBM2HA-GTW and pIGF-GTW vectors using an ATT recombination reaction. Each vector contains an MTT promoter, fluorescent tag, insertion site, and BamHI cut sites. pBM2HA-GTW vector has flanking BTU1 sequences for integration at the BTU1 locus. The black component of the pIGF-GTW vector is rDNA backbone.

An ATT recombination reaction was used to incorporate the mutant tHP1 gene into the HA (pBM2HA-GTW) and GFP (pIGF-GTW) vectors, respectively (Figures 14 and 15). The HA and GFP destination vectors that underwent the ATT recombination reactions and the HA and GFP vectors alone

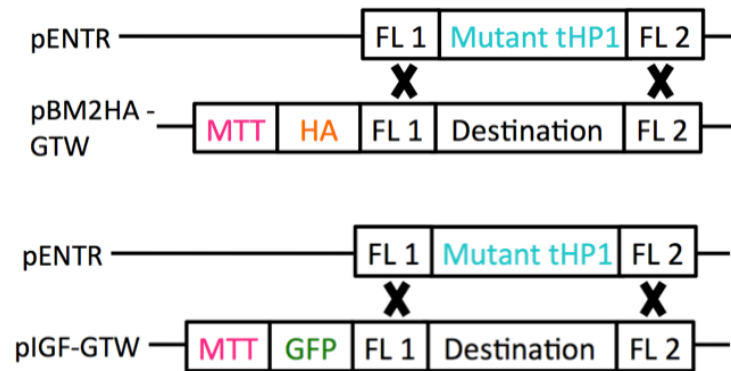


Figure 15. ATT recombination reactions were used to incorporate the mutant tHP1 gene into the pBM2HA-GTW and pIGF-GTW vector with inserted tHP1 gene between ATT recombination sites to create HA and GFP fusion proteins.

were digested with BamHI and the products were visualized using gel electrophoresis to determine whether the mutant gene was successfully incorporated (Figure 16). For the HA vector alone, three bands were expected and observed at 6 kb, 1.3 kb, and 0.7 kb, resulting from three BamHI cut sites in vector (Figure 14). For the HA vector with Site 2 mutant tHP1 successfully recombined, a single band was expected and observed at 12 kb because two of the three BamHI cut sites were lost in the ATT recombination. For the GFP vector alone, six bands were expected at 11.3 kb, 2.2 kb, 2.1 kb, 1.4 kb, 0.7 kb, and 0.4 kb because the vector has five cut sites (Figure 14). Only three bands were observed, including the 11.3 kb band, the 2.2 and 2.1 kb bands together, and the 1.4 kb band. The

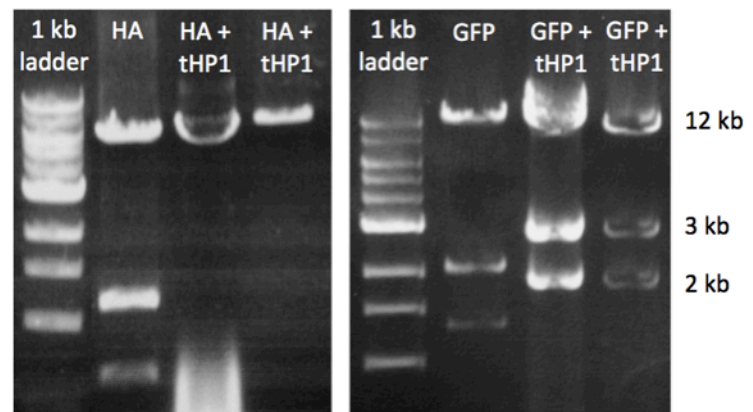


Figure 16. Gel electrophoresis of BAMHI digest reactions with HA vector alone, GFP vector alone, HA vector + tHP1 mutant, and GFP vector + GFP mutant display that tHP1 mutant was successfully incorporated into both vectors.

smaller bands were not observed because they are so small that they do not stain intensely with ethidium bromide or because the ethidium bromide ran off the lower portion of the gel. For the GFP vector with Site 2 mutant tHP1 successfully recombined, bands were expected at 11.3 kb, 3.5 kb, 2.1 kb, 0.7 kb, and 0.4 kb. The recombination results in the addition of 1.2 kb (tHP1 gene and flanking regions) to the 2.2 kb band and the loss of the 1.4 kb band. Bands were observed at 11.3 kb, 3.5 kb, and 2.1 kb. The smaller bands were not observed in this sample either. Thus, gel electrophoresis indicated that the recombination with tHP1 Site 2 was successful and the gene was incorporated into both the HA and GFP vectors.

GFP-tHP1 Site 2 Mutant Vector Successfully Transformed into Tetrahymena

Transformation of the HA and GFP vectors into *Tetrahymena* allows for tagged tHP1 protein expression. The HA vector is integrated into *Tetrahymena*'s BTU1 locus by integrative transformation, whereas the GFP vectors are processed as ribosomal DNA in *Tetrahymena*. The GFP vector is processed alongside *Tetrahymena*'s ribosomal DNA microchromosomes during macronuclear formation in conjugation which allows for large amplification and their subsequent expression (Kapler, 1993).

Four days after the transformation of GFP-tHP1 Site 2 mutant pIGF-GTW vector into *Tetrahymena* by electroporation, one well appeared to have transformant cells after selection

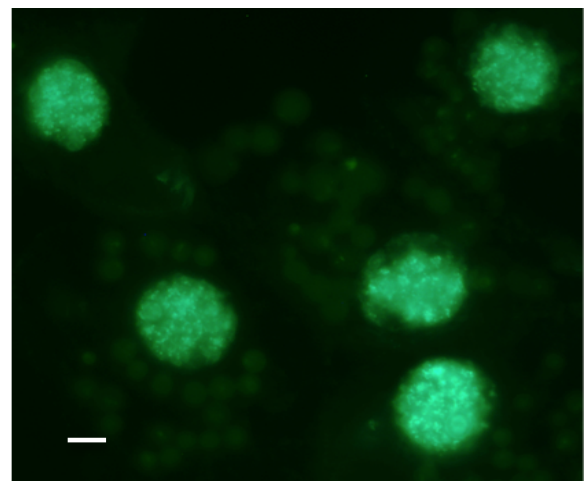


Figure 17. Fluorescence microscopy for GFP reveals that *Tetrahymena* are successfully transformed because they are expressing the GFP protein. Scale bar is 3 microns.

in 90 µg/ml paromomycin media. To determine whether these cells were true transformants, GFP-tHP1 Site 2 mutant expression was induced and examined by fluorescence microscopy. Nuclear GFP expression was observed (Figure 17). Because wild type cells do not express the GFP protein, GFP expression indicated that the vector was successfully transformed into *Tetrahymena*.

GFP-tHP1 Site 2 Mutant Distinctly Localizes to Chromatin Bodies

To examine the effects of hinge region phosphorylation on tHP1 localization within the macronucleus of *Tetrahymena*, additional fluorescence microscopy was utilized. Ultraviolet light

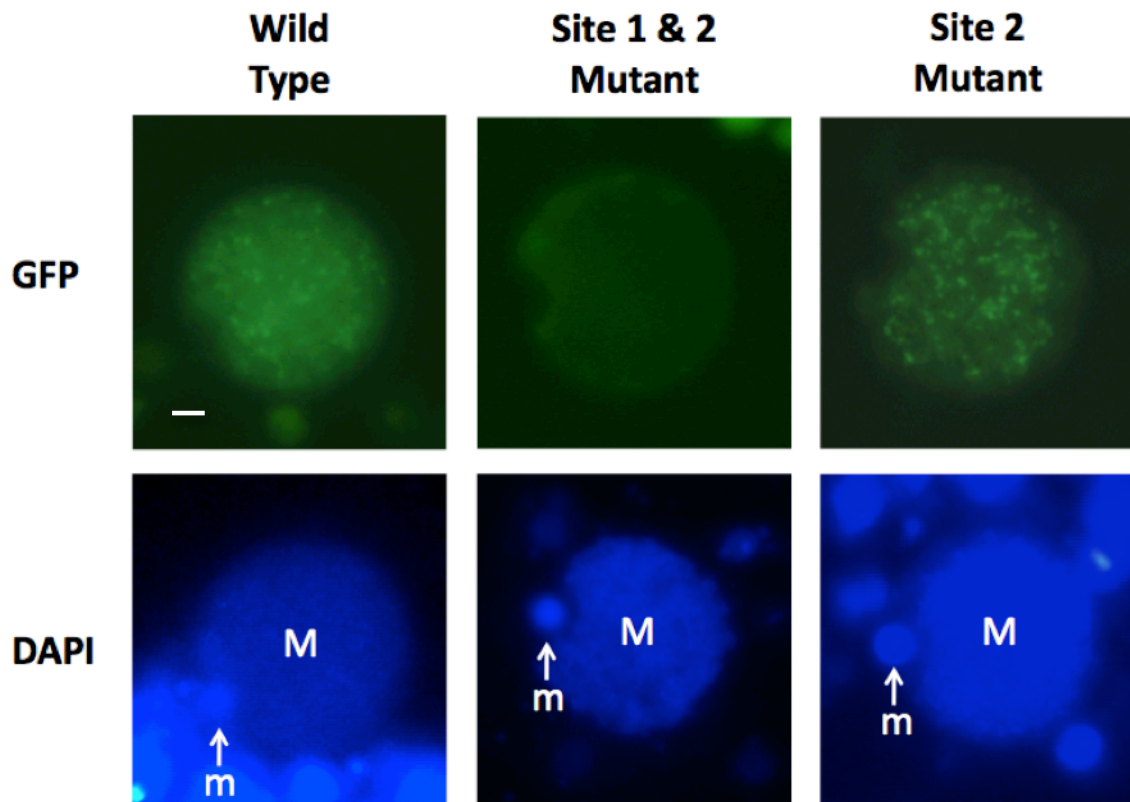


Figure 18. Site 2 mutant shows distinct localization to chromatin bodies. Fluorescence microscopy used to compare GFP-tagged Wild Type tHP1, Site 1 and 2 double mutant tHP1 (T64→A, S102→A, T103→A), and Site 2 tHP1 mutant tHP1 (S102→A, T103→A) localizations. Wild type tHP1 and Site 2 mutant tHP1 display localization to chromatin bodies, whereas Site 1 and 2 double mutant displays a distinct lack of localization to chromatin bodies. DAPI staining used to visualize nuclei (M=macronucleus, m=micronucleus). Scale bar is 1 micron.

was used to visualize the GFP tag on the proteins of interest and to determine the position of the macronucleus and micronucleus via DAPI staining for DNA. We examined the localization of the wild type tHP1 protein, a Site 1 and Site 2 double mutant tHP1 protein, and the Site 2 mutant tHP1 protein (Figure 18). The wild type protein can be phosphorylated at both thr-64 and ser-102/thr-103, the Site 1 and Site 2 double mutant cannot be phosphorylated at either thr-64 or ser-102/thr-103, and the Site 2 mutant can be phosphorylated at thr-64 but not at ser-102 or thr-103.

All three proteins localize solely to the macronucleus. Wild type tHP1 displays a strong localization to the electron-dense, highly compact chromatin bodies within the macronucleus and is not expressed within the micronucleus. These results are consistent with existing knowledge about tHP1 expression and localization within *Tetrahymena*. However, the Site 1 and Site 2 double mutant tHP1 does not display chromatin body localization. Instead, it is diffusely expressed with slightly greater localization to the nuclear envelope than to the interior of the nucleus. This lack of localization to the chromatin bodies suggests that either one or both of the hinge region phosphorylation sites plays an important role in tHP1's localization to regions of heterochromatin. The Site 2 mutant tHP1 displays a strong localization to the chromatin bodies. The extent of the chromatin body localization appears to be comparable to that of wild type tHP1, suggesting that hinge region phosphorylation at Site 2 is not necessary for tHP1 localization to regions of heterochromatin. These results implicate the phosphorylation of the thr-64 residue, not the ser-102/thr-103 residue, as the essential modification that imparts tHP1 with the ability to localize to chromatin bodies.

Bioinformatics Reveals Ten Related Chromo Domain-Containing Proteins in Tetrahymena

To construct a more thorough model of how heterochromatin is formed in *Tetrahymena*,

additional proteins that can bind to markers of heterochromatin must be identified and analyzed, for they may play a role in the process. Because the chromo domain motif is known to recognize, bind to, and facilitate the formation of heterochromatin, analysis of other chromo domain-containing proteins in *Tetrahymena* will likely be beneficial to our understanding of heterochromatin formation. Chromo domain-containing proteins in *Tetrahymena* were identified using bioinformatics techniques. Searching for chromo domains in the TGD revealed several

Table 2. Ten chromo domain containing proteins in *Tetrahymena* were identified by PFam analysis.

Protein Identification	Organism	PFam Score (bits), E-value	CD Location
tHP1 (TTHERM_00705240)	<i>Tetrahymena</i>	47.5, 9.1e-13	5 - 54
CDP1 (Unofficial) (TTHERM_00565610)	<i>Tetrahymena</i>	41.7, 5.8e-11	48 - 97
HPL (Unofficial) (TTHERM_00140850)	<i>Tetrahymena</i>	48.2, 5.5e-13	168 - 215
CDP2 (Unofficial) (TTHERM_00822220)	<i>Tetrahymena</i>	52.8, 2.1e-14	143 - 193
PDD1 (TTHERM_00125280)	<i>Tetrahymena</i>	58.9, 2.5e-16	27 - 79
HPL7 (TTHERM_00551070)	<i>Tetrahymena</i>	46.1, 2.5e-12	17 - 66
HPL5 (TTHERM_00585190)	<i>Tetrahymena</i>	33.6, 2e-08	13 - 54
MBN1 (TTHERM_00585180)	<i>Tetrahymena</i>	37.2, 1.5e-09	15 - 56
CDP3 (Unofficial) (TTHERM_00245410)	<i>Tetrahymena</i>	44.6, 7.3e-12	53 - 100
CHD1 (TTHERM_00193800)	<i>Tetrahymena</i>	35.0, 7.6e-09 31.2, 1.1e-07	189 – 232 706 - 738
HP1a	<i>D. melanogaster</i>	64.7, 4e-18	24 - 71
HP1 α	<i>H. sapiens</i>	66.1, 1.4e-18	20 - 68
Swi6	<i>S. pombe</i>	53.4, 1.3e-14	81 - 133

candidate proteins. When these candidate proteins' amino acid sequences were subjected to PFam analysis, ten had significant PFam chromo domain scores. Table 2 lists each of the *Tetrahymena* proteins, as well as the chromo domain containing proteins from yeast, fruit flies, and humans that are most highly related to tHP1, and the location of their chromo domain(s).

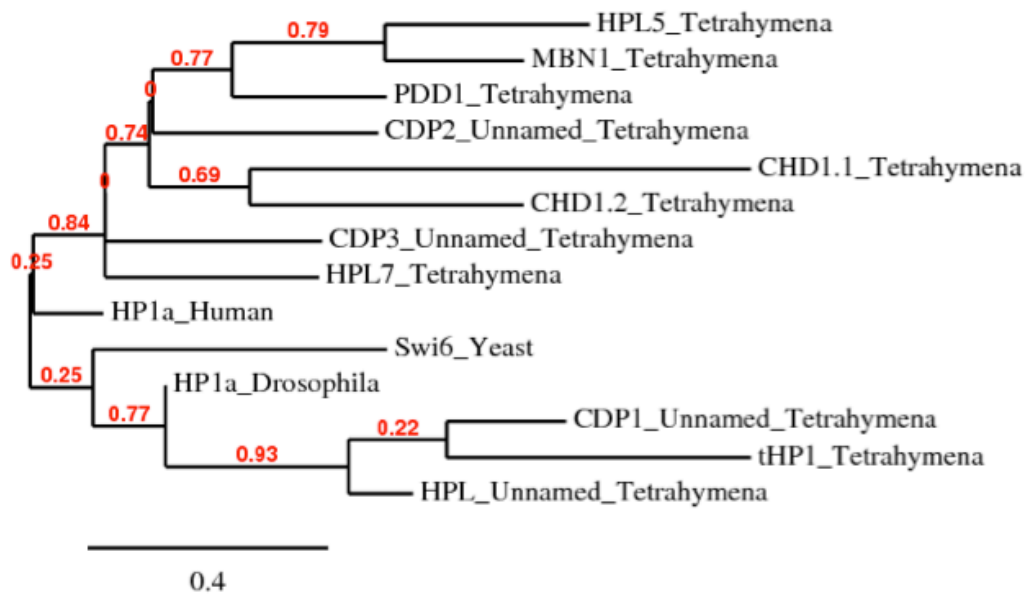


Figure 19. A phylogenetic tree displays three distinct clades among the ten identified chromo domains in *Tetrahymena* and the chromo domains from *D. melanogaster*, *S. Pombe*, and *H. sapiens* tHP1 homologs.

To determine how these chromo domain containing sequences are evolutionarily related, the chromo domain protein sequences revealed by the PFam analysis were isolated, aligned, and formed into a phylogenetic tree (Figure 19). The phylogenetic tree indicates that there are three distinct evolutionary clades among the selected chromo domain-containing proteins. tHP1 is most closely related to a chromo domain protein CDP1 (unofficial name) and to a hypothetical protein HPL (unofficial name). tHP1 is also more closely related to its homologs in *D. melanogaster*, HP1a,

and in *S. Pombe*, Swi6, than it is to the other chromo domain containing proteins within *Tetrahymena* and its *H. sapiens* homolog, HP1 α .

To gain more information about what nuclear processes CDP1 and HPL may be involved in, protein expression profiles for logarithmic growth, starvation, and conjugation were obtained from the Tetrahymena Functional Genome Database (Figure 20).

Expression profiles for CDP1 and HPL were compared with that of tHP1 (Miao, 2001). tHP1 displays the highest expression during logarithmic growth, suggesting that it is involved in the maintenance of heterochromatin. It also is expressed throughout conjugation with peaks from C4 to C6 and at C16. During C4 to C6, conjugating cells undergo meiosis and pronuclear exchange and macronuclear differentiation begins. During this time, chromatin condensation results in chromatin bodies within the macronucleus.

Previous work in the Wiley Lab has

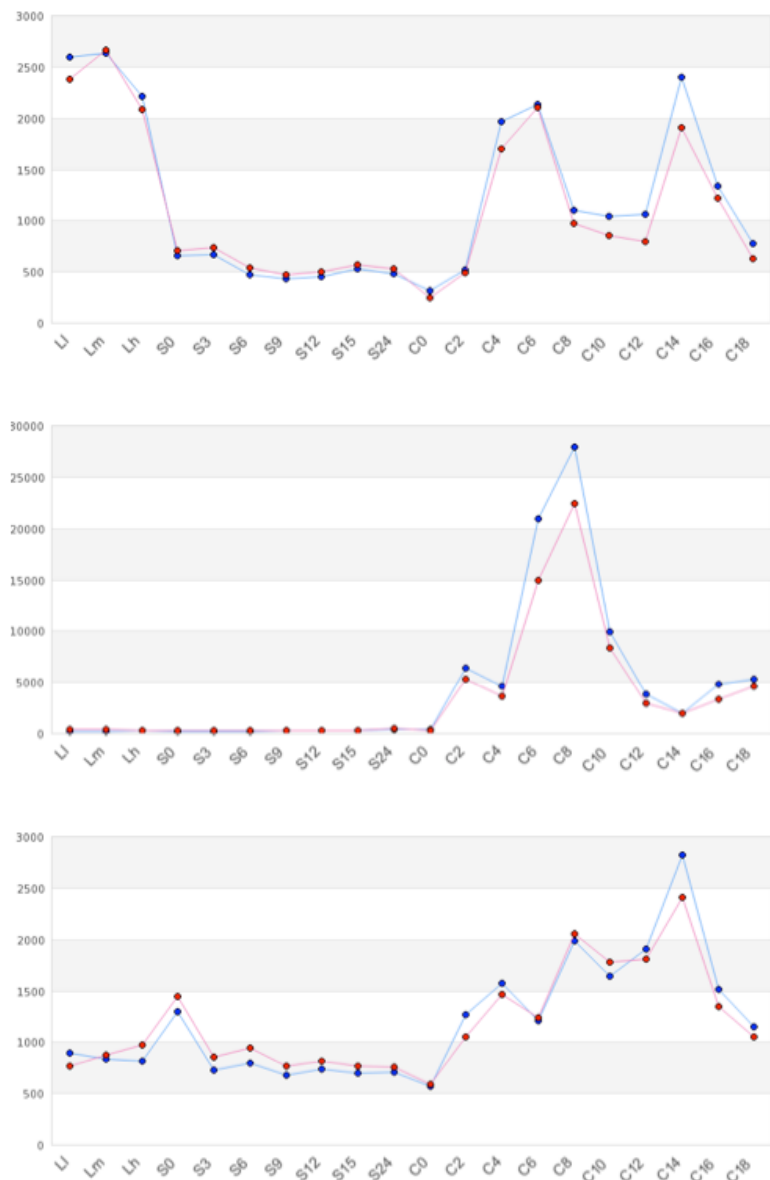


Figure 20. Protein expression profiles for tHP1 (top), CDP1 (middle), and HPL (bottom) during logarithmic growth, starvation, and conjugation. Blue and Red lines correspond to data obtained by Gorovsky and Miao labs in different trials (Miao, 2001).

shown that tHP1 localizes early to the two inactive, differentiating macronuclei (anlagen) to facilitate the formation of chromatin bodies in what will later become a transcriptionally active environment (Katerina Yale, unpublished). The expression profile for tHP1 is consistent with these findings, suggesting that tHP1 plays an important role throughout the major chromatin changes during conjugation. Likewise, CDP1 (unofficial name) and HPL (unofficial name) are expressed during conjugation, suggesting that they, too, play a role in the formation of heterochromatin domains in otherwise transcriptionally active chromatin environments.

Discussion

HP1 is a highly conserved protein that is found in the cell nuclei of nearly all eukaryotic species. HP1 mediates the specific formation of highly condensed domains of chromatin within otherwise transcriptionally active chromatin environments. The formation of heterochromatin is an important cellular process because chromatin condensation results in local gene silencing and increased chromosomal stability. Decreased levels of HP1 expression can lead to a loss of silencing at genes that regulate cancer progression. Decreased levels of HP1 in humans are shown to lead to the development and progression of breast, brain, colon, and ovarian cancers, as well as leukemia and papillary thyroid carcinoma. Low levels of HP1 expression are also one of the best clinical predictors of cancer invasiveness (Dialynas *et al.*, 2008). HP1 also participates in higher-order control mechanisms for telomere length and structure that have important implications for human health and age related diseases. Loss of heterochromatic features at telomeres results in abnormal telomere structure and elongation. These are thought to cause the Telomere Position Effect, in which important neighboring euchromatic genes become repressed (Bauer *et al.*, 2001; Blasco, 2005). These studies provide evidence for the importance of proper heterochromatin formation to human health. Therefore, elucidating how HP1 mediates the formation of heterochromatin is of great scientific and medical interest.

The chromo domain of HP1 plays a critical role in chromatin condensation because it binds directly to regions of heterochromatin by recognizing histone tail methylation (Kwon *et al.*, 2011). Recent scientific literature suggests that the unstructured hinge region of HP1 also plays an important role in HP1 localization to and formation of heterochromatin. The hinge region has been shown to interact nonspecifically with RNA, DNA, and chromatin; however, phosphorylation in the hinge region has been shown to confer stronger and more specific binding properties to HP1 (Zhao

et al., 2000). Mutations that mimic hinge region phosphorylation increase HP1's ability to homodimerize and bind methylated H3K9 (Badugu *et al.*, 2005; Hines, 2006). However, when this phosphorylation is prevented, HP1 displays decreased chromatin binding, homodimerization, affinity for methylated H3K9, and gene silencing (Badugu *et al.*, 2005; Zhao, 2001). In *Tetrahymena* specifically, hyperphosphorylation is correlated with an increase in the size of electron-dense chromatin bodies (Huang *et al.*, 1999). However, tHP1 in *Tetrahymena* is recruited via a methylated H3K9-independent pathway. tHP1 most likely binds to methylated H3K27, a different epigenetic mark of heterochromatin since methylated H3K9 is absent in *Tetrahymena*.

The first main goal in this study is to examine the effects of hinge region phosphorylation on the localization of the HP1-like protein, tHP1, in *Tetrahymena*. The hinge region of tHP1 is known to be multiply phosphorylated at two different sites, Site 1 at thr-64 and Site 2 at ser-102/thr-103. The serine and threonine residues at Site 2 were mutated to alanine to prevent phosphorylation. This Site 2 mutant tHP1 gene was tagged with GFP and placed under the control of an inducible promoter. The mutant tagged gene was transformed into *Tetrahymena* and the protein's localization was visualized by fluorescence microscopy. Fluorescence microscopy was also used to examine the localization of GFP tagged wild type tHP1 and Site 1 and 2 double mutant tHP1.

Fluorescence microscopy revealed that wild type tHP1 and Site 2 mutant tHP1 display distinct localization to chromatin bodies, whereas the Site 1 and 2 double mutant does not display any localization to chromatin bodies (Figure 18). It may be concluded, therefore, that phosphorylation at Site 2 does not influence tHP1 localization to regions of heterochromatin. However, the distinct lack of localization to chromatin bodies displayed by the Site 1 and 2 double mutant tHP1 protein implicates phosphorylation at the thr-64 residue as a key event that results in tHP1's ability to localization to domains of heterochromatin within the macronucleus. tHP1's

localization to regions of heterochromatin may be due to solely to phosphorylation at Site 1 or may be due to a cooperative effect between Site 1 and Site 2 phosphorylation.

A possible mechanism by which hinge region phosphorylation could mediate tHP1 localization is by causing a conformational change in tertiary protein structure (Zhao *et al.*, 2000). It has been shown that the secondary structure of other HP1 proteins is not significantly altered by hinge region phosphorylation because the hinge region is unstructured. However, the tertiary structure of the entire HP1 protein becomes less condensed with increased hinge region phosphorylation (Zhao *et al.*, 2000). The extent of HP1's conformational changes would depend upon the extent to which the hinge region is phosphorylated. The addition of each phosphate would induce an increasingly less condensed protein conformation due to the increasing electrostatic repulsion between the negatively charged phosphoryl groups and the negatively charged DNA backbones in the chromo and chromo shadow domains.

Our speculative model proposes that Site 1 phosphorylation affects tHP1 localization to chromatin bodies because of its proximity to the chromo domain (Figure 21). When Site 1 is phosphorylated, it leads to a conformational change in tHP1 that causes the protein to assume a less

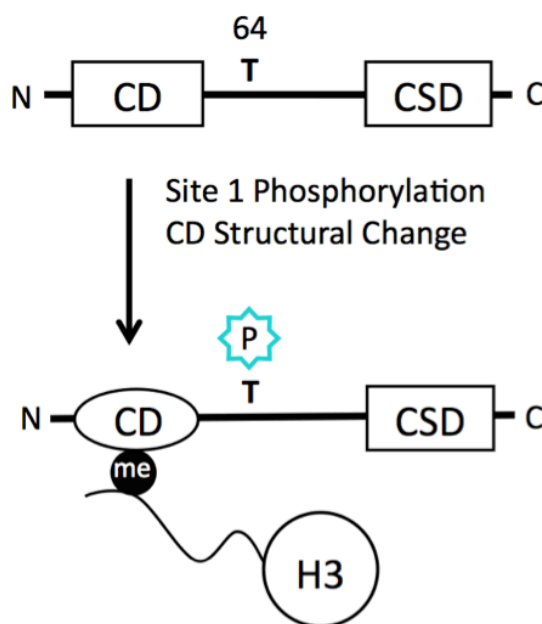


Figure 21. Proposed model for effect of Site 1 hinge region phosphorylation on the chromo domain's ability to recognize and bind marks of heterochromatin. Phosphorylation results in a conformational change in the chromo domain that increases tHP1's binding affinity for methylated H3K27 residues.

condensed conformation. This, in turn, can influence the upstream chromo domain structure and function by making the chromo domain more easily accessible. This change is predicted to enhance abilities to recognize and bind to regions of heterochromatin to the chromo domain. Specifically, we think that it improves the chromo domain's ability to recognize and bind methylated H3K27, *Tetrahymena's* primary epigenetic mark of heterochromatin. In all other eukaryotic cells, heterochromatin formation is mediated by HP1 binding to methylated H3K9; however, heterochromatin in *Tetrahymena* does not possess methylated H3K9 residues. Just as HP1 is recruited to regions of heterochromatin through methylated H3K9-dependent mechanisms in most eukaryotes, it is predicted that tHP1 is recruited to regions of heterochromatin through a related methylated H3K27-dependent mechanism.

To gain a more complete understanding of the effect of hinge region phosphorylation on the localization of tHP1, the GFP tagged Site 1 mutant tHP1 must be transformed into *Tetrahymena*. This will allow for a more direct examination of the effect of Site 1 phosphorylation on tHP1 localization and may provide evidence that allows us to modify our proposed model (Figure 20). HA tagged versions of each tHP1 protein (wild type, Site 1 mutant, Site 2 mutant, and Site 1 and Site 2 mutant) should also be transformed into *Tetrahymena*. This transformation has proven difficult, and, if the biolistic transformation gun continues to fail, the task will be completed by an out-of-state colleague. The HA tag would allow for confirmation of our protein localization results by using immunofluorescence staining and would also facilitate protein isolation and purification.

Determining the localization of each form of tHP1 through different stages of conjugation using both the GFP and HA tagged forms should be pursued. The tHP1 expression profile suggests that tHP1 plays an important role throughout the major chromatin changes during conjugation and is particularly active during chromatin body condensation of the new macronuclei (Figure 19). The

ability to isolate each form of tHP1 during key events in the conjugation process would allow us to determine the levels of phosphorylation using an acid urea gel (as in Figure 7).

An experimentally difficult control study should also be conducted to verify our results. The four different GFP and HA tagged proteins should be expressed in tHP1 knockout cells. Loss of tHP1 is not lethal to *Tetrahymena*, and induced expression of our proteins in the absence of background wild type tHP1 expression would provide definitive information about the localization and function of each form of the protein.

The second main goal of this research was to identify other tHP1-like proteins in *Tetrahymena* with the hopes of developing a more thorough model of how heterochromatin is formed. The chromo domain is known to recognize and bind to regions of heterochromatin, so it is predicted that other chromo domain-containing proteins may work alongside tHP1 to construct these condensed domains. Proteins with chromo domains are also known to play a role on chromatin remodeling (Kwon *et al.*, 2011). Using bioinformatics techniques, ten chromo domain-containing proteins were identified and an analysis of their evolutionary relationships revealed that there are two currently unstudied proteins that are highly related to tHP1, CDP1 (unofficial name) and HPL (unofficial name).

Protein expression profiles indicate that CDP1 and HPL are both expressed during conjugation (Figure 19). Conjugation entails a highly dynamic chromatin environment in which nuclear differentiation results in condensation and decondensation of chromatin. Because the chromo domain sequences in CDP1 and HPL are highly related to that of tHP1 and are very active during dynamic chromatin changes, further investigations hold promise for improving our current models of heterochromatin formation in *Tetrahymena*. Previous work in the Wiley Lab demonstrates that tHP1 quickly localizes to the newly differentiated, transcriptionally active

macronucleus to form regions of transcriptionally inactive chromatin (Katerina Yale, unpublished).

The close evolutionary relationships and similar expression patterns of CDP1 and HPL to tHP1 indicate that these three chromo domain-containing proteins may be part of a greater family of proteins that are involved in the establishment and maintenance of regions of heterochromatin. The sequence similarity also raises the possibility that they may be regulated in similar ways to tHP1. Perhaps these proteins all respond to similar intracellular and extracellular signals that result in their phosphorylation and subsequent participation in chromatin formation.

Partnering with the Horrell Lab to characterize the functions of these proteins will be worthwhile. Horrell has identified other chromo domain-containing proteins in *Tetrahymena* and is using gene expression profiles as a starting point from which to design experiments that investigate the localization and function of the proteins of interest. The lab is obtaining compelling results, as they have identified interactions between PDD1 and HPL1 that mediate anlagen formation during conjugation (Horrell, unpublished).

Bioinformatics work also revealed that tHP1 is highly similar to *S. pombe*'s Swi6 and *Drosophila*'s HP1a. Both of these proteins have been widely studied and play important regulatory roles in gene expression. The close, highly conserved relationship between the chromo domains in tHP1 and Swi6 is expected because both *Tetrahymena* and *S. pombe* are single celled eukaryotes. Furthermore, tHP1's close relationship to *Drosophila* HP1a than to other chromo domain-containing proteins in *Tetrahymena* is important. It is generally accepted that methylated H3K9 stabilizes HP1a binding to chromatin and HP1a subsequently forms a dimer that links adjacent nucleosomes to condense chromatin. Meanwhile, HP1a also interacts with histone methyl transferases (HMTs), which result in nearby H3K9 methylation that causes HP1a to spreading and heterochromatin propagation. While this model accurately represents centromeric HP1a activity, a recent study

reports that HP1a can also bind to promoters of active genes all along highly repressed chromosome four independently of methylated H3K9 to induce heterochromatin formation and spreading (Figueiredo *et al.*, 2012). Because the chromo domain is responsible for methylated H3K9 binding, it is interesting that both tHP1 and HP1a have related chromo domains and are the only known HP1 proteins to be recruited via methylated H3K9-independent mechanisms. As *Tetrahymena*'s chromosomes do not have centromeres, tHP1 may share a similar mechanism to noncentromeric HP1a recruitment along chromosome four. As we have found in *Tetrahymena*, the methylated H3K9-independent mechanism by which heterochromatin is formed in *Drosophila* may be partially mediated by HP1 hinge region phosphorylation.

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